Inhibition of *Clostridium perfringens* Spore Germination and Outgrowth by Lemon Juice and Vinegar Product in Reduced NaCl Roast Beef

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Abstract: Inhibition of *Clostridium perfringens* spore germination and outgrowth in reduced sodium roast beef by a blend of buffered lemon juice concentrate and vinegar (MoStatin LV1) during abusive exponential cooling was evaluated. Roast beef containing salt (NaCl; 1%, 1.5%, or 2%, w/w), blend of sodium pyro- and poly-phosphates (0.3%), and MoStatin LV1 (0%, 2%, or 2.5%) was inoculated with a 3-strain *C. perfringens* spore cocktail to achieve final spore population of 2.5 to 3.0 log CFU/g. The inoculated products were heat treated and cooled exponentially from 54.4 to 4.4 °C within 6.5, 9, 12, 15, 18, or 21 h. Cooling of roast beef (2.0% NaCl) within 6.5 and 9 h resulted in <1.0 log CFU/g increase in *C. perfringens* spore germination and outgrowth, whereas reducing the salt concentration to 1.5% and 1.0% resulted in >1.0 log CFU/g increase for cooling times longer than 9 h (1.1 and 2.2 log CFU/g, respectively). Incorporation of MoStatin LV1 into the roast beef formulation minimized the *C. perfringens* spore germination and outgrowth to <1.0 log CFU/g, regardless of the salt concentration and the cooling time.

Keywords: abusive cooling, *Clostridium perfringens*, lemon juice, outgrowth, vinegar

Practical Application: Cooked, ready-to-eat meat products should be cooled rapidly to reduce the risk of *Clostridium perfringens* spore germination and outgrowth. Meat processors are reducing the sodium chloride content of the processed meats as a consequence of the dietary recommendations. Sodium chloride reduces the risk of *C. perfringens* spore germination and outgrowth in meat products. Antimicrobials that contribute minimally to the sodium content of the product should be incorporated into processed meats to assure food safety. Buffered lemon juice and vinegar can be incorporated into meat product formulations to reduce the risk of *C. perfringens* spore germination and outgrowth during abusive cooling.

Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium. It is ubiquitous in the environment and is a frequent contaminant of raw protein foods of animal origin. *C. perfringens* prevalence rate of 30% to 80% have been reported in raw and processed foods (Lin and Labbe 2003), with beef products responsible for 40% of *C. perfringens* foodborne outbreaks (Bhunia 2008). The food poisoning is caused by *C. perfringens* enterotoxin mainly produced by type A strains (Bhunia 2008). It is estimated that 965958 cases of *C. perfringens* gastrointestinal illness occur annually in the U.S., ranking 3rd as the most common cause of foodborne illness (Centers for Disease Control and Prevention 2011).

Meat and poultry products are associated with the majority of *C. perfringens* outbreaks (Linch and others 2006; McClane 2007). Improper cooling of meat products and holding at abusive temperatures are recognized as the most common causes since heat-activated spores that survive thermal processing can germinate and grow rapidly under abusive temperature conditions. Germination and outgrowth of *C. perfringens* spores during improper cooling of thermally processed meat products has been reported (Juneja 1994; Sabah and others 2004). The growth of *C. perfringens* has been used as performance standard by the U.S.D.A. Food Safety and Inspection Service (FSIS) and the U.S. Food and Drug Administration (FDA) to assess the safety of cooling process for meat, poultry, and foods containing meat and poultry (USDA-FSIS 2001; US FDA 2001). The FDA Division of Retail Food Protection (2001) established a guideline that all ready-to-eat (RTE) meat products be cooled from 60 to 21 °C in 2 h and from 21 to 5 °C in 4 h. The FSIS compliance guidelines for cooling of thermally processed meat and poultry products (2001) recommend that cooling from 54.4 to 26.7 °C should be achieved within 1.5 h and subsequently from 26.7 to 4.4 °C within 5 h. In case the cooling guidelines cannot be achieved, these products should be cooled at a rate sufficient to prevent more than a 1 log increase of *C. perfringens*.

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Salt (NaCl) is a major source of sodium in the diet. The Dietary Guidelines for Americans (U.S. Department of Agriculture and U.S. Department of Health and Human Services 2010) recommend sodium intake of no more than 2300 mg per day for individuals of ≥2 years of age. However, the average American consumes more than 3400 mg of sodium per day, which is 1.5 times of the recommended amount (Henney and others 2010). High intake of sodium has been related to prevalence of high blood pressure (hypertension) and related diseases such as cardiovascular and kidney disease, and stroke (Strazzullo and others 2009; Gradual and others 2012). It is estimated that more than 80% of sodium intake is related to consumption of processed food, with 20% from meat and poultry products. Therefore, reduction of sodium in meat and poultry products can play an important role in reducing the incidence of high blood pressure and related diseases.

Organic acids and sodium and potassium salts of lactic, acetic, citric, pyruvic, and propionic acids are generally recognized as safe (GRAS) and are extensively used to enhance flavor and improve microbial quality of meat and poultry products. The USDA-FSIS (2000) has approved the use of sodium acetate, sodium diacetate, sodium lactate, and potassium lactate in meat and poultry products as antimicrobial ingredients to control *Listeria monocytogenes*. Several studies have evaluated the inhibitory effect of organic acid salts on the germination and outgrowth of *C. perfringens* spores. Juneja and Thippareddi (2003) reported that 1% of sodium lactate and sodium acetate were sufficient to control the growth of *C. perfringens* during 15 h extended cooling in marinated ground turkey breast, and that 1% of buffered sodium citrate inhibited the growth up to 21 h of cooling. Addition of calcium lactate (≥2%), potassium lactate, or sodium lactate (≥3%) meets USDA-FSIS performance standard to control *C. perfringens* growth to <1 log CFU/g in injected pork (Velugoti and others 2007).

The growing demand for natural and organic foods from consumers has resulted in a search for natural antimicrobials for use in meat and poultry products. The efficacy of natural antimicrobials against the growth of *C. perfringens* has been reported in literature. Juneja and others (2005) reported that 200 ppm grapefruit extract inhibited the growth of *C. perfringens* in marinated *sous-vide* chicken products at 19 and 25 °C. Incorporation of carvacrol, cinnamaldehyde, thymol, or oregano oil into cooked ground beef inhibited *C. perfringens* spore germination and outgrowth during exponential cooling within 12 h (Juneja and others 2006). Oregano powder in combination with organic acid salts has been reported to inhibit the growth of *C. perfringens* during cooling of *sous vide* cooked ground beef products (Sabah and others 2004).

Lemon juice and vinegar, the natural source of citric acid and acetic acid, respectively, are likely to have inhibitory effect on the growth of *C. perfringens* according to previous studies on organic acid salts (Juneja and Thippareddi 2003; Velugoti and others 2007). However, their juices cannot be used in meat and poultry products because of the accompanying undesirable organoleptic property changes and protein denaturation resulting in reduced binding of meat matrix. Nevertheless, buffered counterparts of organic acids or their salts can be used with minimal impact on meat quality.

There is a growing trend in the development of reduced sodium meat and poultry products due to the health concerns. However, sodium chloride is an important antimicrobial in meat, its reduction significantly challenges meat safety, particularly for the abusive cooled products. Therefore, alternative natural antimicrobials are required to assure safety of reduced salt meat and poultry products. The inhibitory effect of a blend of buffered lemon juice and vinegar on germination and outgrowth of *C. perfringens* spores in reduced sodium roast beef was evaluated during abusive cooling.

Materials and Methods

Bacterial cultures

Three individual enterotoxin-producing strains of C. *perfringens* were used in this study: NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13). Additional information about these strains and their maintenance has been reported elsewhere (Juneja and others 1993).

Preparation of spore cocktail

A 0.1 mL aliquot of stock culture was inoculated into 10 mL of freshly prepared fluid thioglycollate medium (FTM; BBL, Sparks, Md., U.S.A.). Inoculated media were heat shocked for 20 min at 75 °C in a waterbath (Isotemp 3013H, Fisher Scientific, Pittsburgh, Pa., U.S.A.), cooled in cooled water, and incubated for 18 h at 37 °C. A 1.0 mL portion of this culture was transferred to 10 mL of freshly steamed FTM and then incubated for 4 h at 37 °C and the activation procedure was repeated 2 times. Contents of FTM were transferred to 100 mL of modified Duncan Strong medium (DS) and incubated for 24 h at 37 °C aerobically under stationary conditions. The original DS formulation was modified by replacing starch with 0.4% raffinose (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and supplemented with 100 mg/L of caffeine (Sigma) to enhance sporulation. The cultures of each strain were harvested by centrifugation at 7012 × *g* for 20 min at 4 °C (Beckman, J2-HS) and washed twice using 50 mL of sterile distilled water. The spore crop of each strain was stored separately at 4 °C for up to 6 mo until used. A spore cocktail containing all 3 strains of *C. perfringens* was prepared immediately before experiments by mixing equivalent numbers of spores from each suspension.

Meat preparation

Fat-trimmed beef top rounds (85% to 90% lean) from Loeffel Meat Laboratory in the Department of Animal Science, Univ. of Nebraska–Lincoln were ground through a 2.54 cm grinder plate (Hobart, Troy, Ohio, U.S.A.), and reground through a 0.16 cm grinder plate. The ground meat (453 g) was placed in bags (3-mil standard barrier nylon vacuum pouch with a water vapor transmission rate of 10 g/L/m^2/24 h at 37.8 °C and 100% relative humidity and an oxygen transmission rate of 3000 cm^3/L/m^2/24 h at 23 °C and 1 atm; Prime Source, Kansas City, Mo., U.S.A.), vacuum packaged (A300/H, Multivac, Wolfertschweden, Germany), and stored at −20 °C until use. Salt (NaCl; 1.0, 1.5, 2.0, w/w), 0.3% of sodium pyro- and poly-phosphates blend (Brifisol® 85 Instant, BK Guilini, Semi Valley, Calif., U.S.A.) and blend of lemon juice concentrate and vinegar (MoStatin LV1; Isoage Technologies, Athens, Ga., U.S.A.) at concentrations of 0%, 2.0%, and 2.5% were dissolved in water and added to the raw beef. The meat was separately mixed for 2 min in a kitchen mixer (Model K5SSWH, KitchenAid, Troy, Ohio, U.S.A.). Five-gram portions of each treated sample were weighed into vacuum pouches, measuring 6.35 by 12.7 cm (2.5 in. × 5 in.) (Prime Source, Kansas City, Mo., U.S.A.), vacuum sealed at 1.2 kPa using a vacuum-packaging machine (A300/H, Multivac, Wolfertschwenden, Germany), and frozen at −20 °C until use.

Spore inoculation, heat, and cooling procedures

Samples were thawed overnight in a refrigerator at 5 °C, and each pouch of meat was aseptically inoculated with 100 μL of the 3-strain spore cocktail of *C. perfringens* to attain a final spore

Table 1–Mean pH and water activity (a_w) values of roast beef containing different combinations of sodium chloride and MoStatin LV1.

Salt (%)	MoStatin LV1 (%)	pH[a]	a_w[a]
2.0	0.0	5.79 ± 0.04	0.980 ± 0.001
	2.0	5.72 ± 0.04	0.977 ± 0.003
	2.5	5.71 ± 0.04	0.976 ± 0.001
1.5	0.0	5.76 ± 0.04	0.982 ± 0.001
	2.0	5.73 ± 0.04	0.979 ± 0.000
	2.5	5.72 ± 0.03	0.979 ± 0.001
1.0	0.0	5.73 ± 0.07	0.986 ± 0.002
	2.0	5.75 ± 0.02	0.982 ± 0.001
	2.5	5.75 ± 0.02	0.981 ± 0.003

[a]Values are averages of 3 independent replicates ± standard deviations; values for each sample were measured in triplicate.

population of ca. 2.5 to 3.0 log CFU/g of meat. The inoculated pouches were vacuum sealed as described above, massaged manually for 30 s to evenly distribute spores, and flattened to a uniform thickness. Two pouches from each treatment were submerged in a water bath (RTE 740, ThermoNeslab, Portsmouth, N.H., U.S.A.) to heat from 4.4 to 71.1 °C in 9.75 h, cool to 57.1 °C in 2.75 h and to 54.4 °C within 5 min. After heat activation, 1 of the 2 pouches was cooled immediately in an ice water bath, and used for the enumeration of C. *perfringens*. The other pouch was then cooled exponentially from 54.4 to 4.4 °C within 6.5, 9, 12, 15, 18, or 21 h.

C. perfringens population in the meat samples were enumerated following the protocol described by Valenzuela-Martinez and others (2010) and expressed as log CFU/g of meat.

Measurement of pH and water activity

Five-gram portions of the noninoculated samples were homogenized with 20 mL deionized water for 2 min in the stomacher (Stomacher 400, Seward Medical, London, U.K.). The pH was measured using a combination electrode (Accumet13–620-108A, Fisher Scientific) and a pH meter (Accumet-Basic/AB 15, Fisher Scientific) by placing the probe in the sample homogenate. The water activity (a_w) was measured using Aqua Lab 3TE water activity meter (Decagon Devices Inc., Pullman, Wash., U.S.A.).

Statistical analyses

Three independent replications were performed for each of the 6 exponential cooling rates. Data were analyzed and compared with analysis of variance of the general linear model procedure of the statistical analysis system (SAS Inst., Cary, N.C., U.S.A.). Fishers' least significant difference ($\alpha = 0.05$) was used to separate means of the *C. perfringens* population.

Results and Discussion

The pH and a_w of roast beef (control, 2% NaCl) were 5.79 and 0.980, respectively (Table 1). Incorporation of buffered lemon juice and vinegar did not affect ($P > 0.05$) the pH of roast beef. This is probably due to the buffering capacity of the meat and the relatively high pH of MoStatin LV1 (5.65). However, several factors such as meat species, other ingredients (such as phosphates) and their concentrations in meat affect the pH of the product. For example, Valenzuela-Martinez and others (2010) reported a decrease in pH of ground turkey roast by buffered lemon juice and vinegar. Whereas, Juneja and Thippareddi (2003) observed

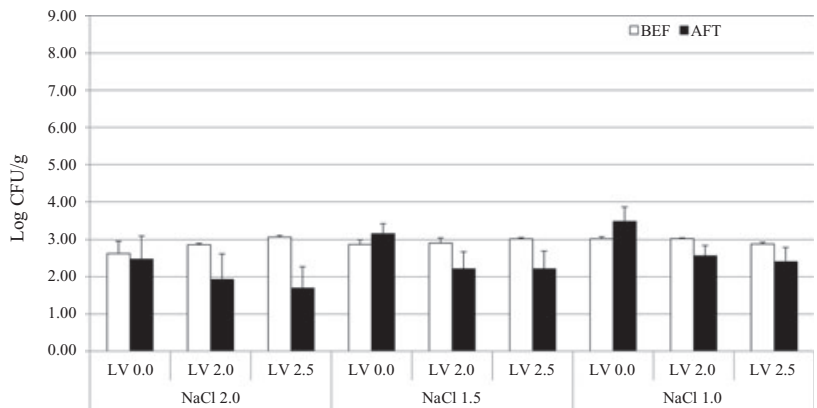

Figure 1–Mean populations of *Clostridium perfringens* in roast beef before (□) and after exponentially cooling from 54.4 to 4 °C in 6.5 h (■). MoStatin LV1 (LV) was added at concentrations of 0%, 2.0%, and 2.5% NaCl was added at concentrations of 1.0%, 1.5%, and 2.0%.

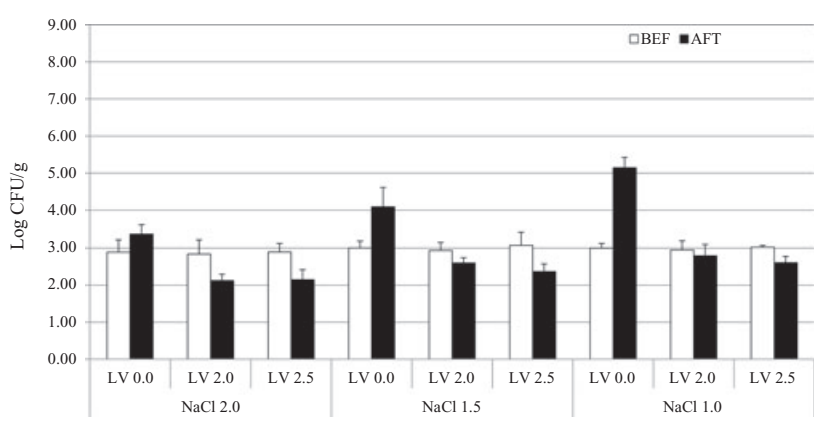

Figure 2–Mean populations of *Clostridium perfringens* in roast beef before (□) and after exponentially cooling from 54.4 to 4 °C in 9 h (■). MoStatin LV1 (LV) was added at concentrations of 0%, 2.0%, and 2.5% NaCl was added at concentrations of 1.0%, 1.5%, and 2.0%.

that the addition of buffered sodium citrate increased the pH of roast beef from 5.62 to 5.78, while it reduced ($P > 0.05$) the pH of injected pork from 6.11 to 6.07.

The a_w of control roast beef containing 1% NaCl was higher than that of the samples containing 1.5% or 2% NaCl. Although the addition of MoStatin LV1 slightly reduced the a_w of the samples, the minimal decreases in a_w due to the addition of MoStatin LV1 should have minimal effect on the growth of *C. perfringens* considering that the growth of *C. perfringens* is not likely inhibited by $a_w > 0.97$ (Labbe and Juneja 2006).

Cooling of roast beef within 6.5 h from 54.4 to 4.4 °C resulted in increases (0.47 and 0.29 log CFU/g) of *C. perfringens* population in control samples containing 1% and 1.5% NaCl (Figure 1). Valenzuela-Martinez and others (2010) reported a 0.51 log CFU/g increase of *C. perfringens* population in ground turkey breast during 6.5 h of cooling from 54.4 to 4.0 °C; and Velugoti and others (2007) also reported a minimal increase of 0.41 log CFU/g in injected pork. These results validate the adequacy of USDA-FSIS compliance guidelines for cooling of thermally processed meat and poultry products. The incorporation of MoStatin LV1 slightly reduced *C. perfringens* population in roast beef. For example, 2.5% MoStain LV resulted in 0.47, 0.80, and 1.37 log CFU/g reduction of *C. perfringens* populations in roast beef containing 1.0%, 1.5%, and 2.0% NaCl, respectively.

Abusive cooling can occur due to inadequate cooling capacity, malfunction of the equipment, or power failures. During the 9 h cooling, >1 log CFU/g increases (1.09 and 2.14 log CFU/g, respectively) of *C. perfringens* populations were observed in the control roast beef containing 1.0% and 1.5% NaCl (Figure 2). Reducing salt content in meat can result in a greater risk of *C. perfringens* growth in case of abusive cooling or holding of the products. Extending the cooling to 9 h beyond the USDA-FSIS recommended cooling regime (6.5 h) did not result in a >1 log CFU/g increase of *C. perfringens* growth in roast beef (2.0% NaCl). While a minimal increase (<1 log CFU/g) of *C. perfringens* growth was observed in roast beef, caution should be exercised that the result may not necessarily apply to other meat and poultry products, as the germination and outgrowth of *C. perfringens* varies in different meat substrates and other ingredients added to the meat. For example, a >2 log CFU/g increase of *C. perfringens* population following 9 h cooling was observed in ground turkey roast and in pork (Singh and others 2010; Valenzuela-Martinez and others 2010). The incorporation of MoStatin LV1 reduced the *C. perfringens* populations, although the effect was minimal in the reduced sodium roast beef (1.0% and 1.5% NaCl).

The mean *C. perfringens* populations reached to 4.88, 6.37, and 6.57 log CFU/g for 12 h cooling of the control samples containing 2.0%, 1.5%, and 1.0% of NaCl, respectively (Figure 3). Comparing the cooling times of 12 and 15 h, MoStatin LV1 resulted in larger reductions of *C. perfringens* populations during longer cooling time (Figure 3 and 4). This finding is similar to the results of Juneja and Thippareddi (2003) and Velugoti and others (2007) on the inhibitory effect of sodium citrate in meat products. The authors hypothesized that the antimicrobial activity of sodium citrate was temperature dependent, with greater *C. perfringens* population reductions being observed with longer exposures to higher

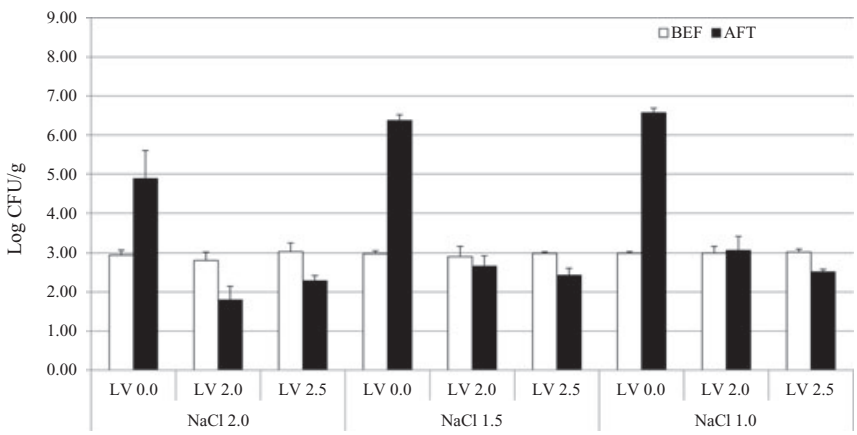

Figure 3–Mean populations of *Clostridium perfringens* in roast beef before (□) and after exponentially cooling from 54.4 to 4 °C in 12 h (■). MoStatin LV1 (LV) was added at concentrations of 0%, 2.0%, and 2.5% NaCl was added at concentrations of 1.0%, 1.5%, and 2.0%.

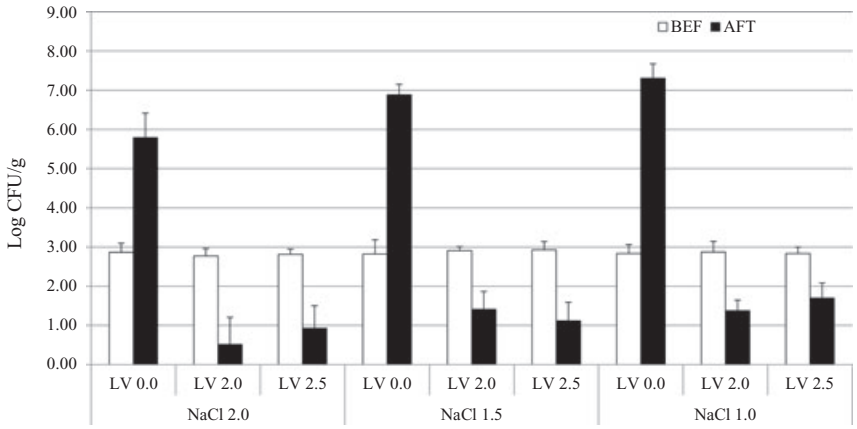

Figure 4–Mean populations of *Clostridium perfringens* in roast beef before (□) and after exponentially cooling from 54.4 to 4 °C in 15 h (■). MoStatin LV1 (LV) was added at concentrations of 0%, 2.0%, and 2.5% NaCl was added at concentrations of 1.0%, 1.5%, and 2.0%.

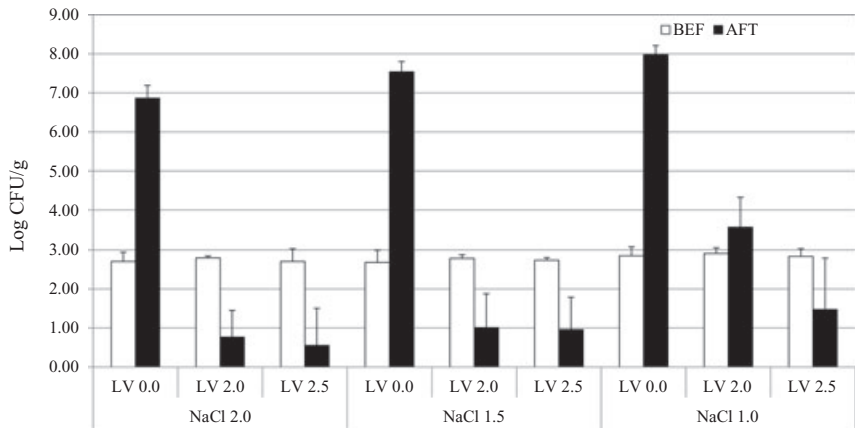

Figure 5–Mean populations of *Clostridium perfringens* in roast beef before (□) and after exponentially cooling from 54.4 to 4 °C in 18 h (■). MoStatin LV1 (LV) was added at concentrations of 0%, 2.0%, and 2.5% NaCl was added at concentrations of 1.0%, 1.5%, and 2.0%.

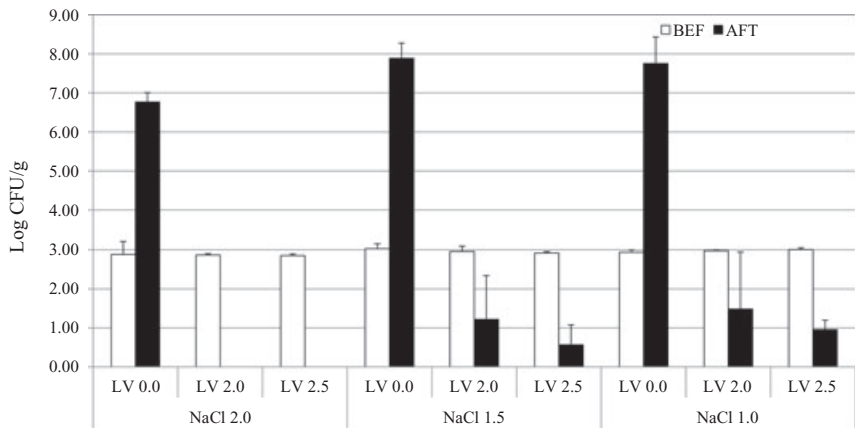

Figure 6–Mean populations of *Clostridium perfringens* in roast beef before (□) and after exponentially cooling from 54.4 to 4 °C in 21 h (■). MoStatin LV1 (LV) was added at concentrations of 0%, 2.0%, and 2.5% NaCl was added at concentrations of 1.0%, 1.5%, and 2.0%.

temperatures. Accordingly, citrate-a major component of MoStatin LV1 may account for the larger *C. perfringens* reductions during longer cooling times. As to acetate, the other active ingredient of MoStatin LV1, whether its antimicrobial effect is also temperature dependent needs further study.

The antimicrobial activity of organic acids has been well established. They are effective at pH values below their pK_a in the undissociated state. The undissociated molecule, upon entering the microbial cell, reduces the intracellular pH and alters cell membrane permeability by affecting substrate transport and inhibiting electron transport necessary for energy generation (Beuchat and Golden 1989). Furthermore, citric acid and phosphates are good chelating agents in meat and can effectively bind metal ion required for *C. perfringens* growth (Akhtar and others 2008). After cooking, free iron is released from the myoglobin and hemoglobin of meat (Lombardi-Boccia and others 2002), serving as an important nutrient in microbial metabolism. The chelation of free irons by citric acid and phosphates would inhibit the microbial growth. Singh and others (2010) reported that the antimicrobial effect of phosphate in meat can be attributed to the chelation of metal ions in addition to the reduction of pH when acid phosphates are used.

Approximately 5 log increases in *C. perfringens* population in control samples containing 1.5% and 1.0% NaCl were observed, when the cooling times were extended to 18 and 21 h (Figure 5 and 6). During 21 h cooling, *C. perfringens* populations were reduced to below the detection limit (0.70 log CFU/g) in the product containing 2% NaCl, regardless of the concentration of MoStatin LV1. *C. perfringens* in the reduced sodium roast beef were still viable and detectable on TSC agar. The *C. perfringens* population was MoStatin LV1 concentration dependent, with lower population observed in product containing higher MoStatin LV1 concentration. Nevertheless, incorporation of MoStatin LV1 into the product resulted in *C. perfringens* growth below that observed in control product for all the abusive cooling times.

Conclusion

Sodium chloride is widely used in processed meat as flavor enhancer and as an antimicrobial agent. In this study, 2% NaCl effectively prevented the germination and outgrowth of *C. perfringens* spores in ground, roast beef within 9 h of (abusive) cooling. Reducing sodium concentration in the roast beef resulted in greater increases ($P \leq 0.05$) in *C. perfringens* population. The blend of buffered lemon juice concentrate and vinegar was effective in controlling germination and outgrowth of *C. perfringens* spores in ground roast beef during abusive cooling times regardless of NaCl content. The incorporation of this natural antimicrobial agent would probably apply to other ready-to-eat meat and poultry products, which enables the reduction of NaCl content and provides an additional hurdle for microbial growth and enhances meat safety during abusive cooling. However, caution should be exercised in extrapolating data obtained from this study to other meat products, since the variation of meat substrates and other ingredients may affect the inhibition of *C. perfringens* germination and outgrowth by MoStatin LV1.

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